Agonist-Specific Coupling of a Cloned *Drosophila melanogaster* D1-Like Dopamine Receptor to Multiple Second Messenger Pathways by Synthetic Agonists

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The mechanism of coupling of a cloned *Drosophila* D1-like dopamine receptor, DopR99B, to multiple second messenger systems when expressed in *Xenopus* oocytes is described. The receptor is coupled directly to the generation of a rapid, transient intracellular Ca^{2+} signal, monitored as changes in inward current mediated by the oocyte endogenous Ca^{2+}-activated chloride channel, by a pertussis toxin-insensitive G-protein-coupled pathway. The more prolonged receptor-mediated changes in adenylyl cyclase activity are generated by an independent G-protein-coupled pathway that is pertussis toxin-sensitive but calcium-independent, and $G_{\beta\gamma}$-subunits appear to be involved in the transduction of this response. This is the first evidence for the direct coupling of a cloned D1-like dopamine receptor both to the activation of adenylyl cyclase and to the initiation of an intracellular Ca^{2+} signal. The pharmacological profile of both second messenger effects is identical for a range of naturally occurring catecholamine ligands (dopamine > norepinephrine > epinephrine) and for the blockade of dopamine responses by a range of synthetic antagonists. However, the pharmacological profiles of the two second messenger responses differ for a range of synthetic agonists. Thus, the receptor exhibits agonist-specific coupling to second messenger systems for synthetic agonists. This feature could provide a useful tool in the genetic analysis of the roles of the multiple second messenger pathways activated by this receptor, given the likely involvement of dopamine in the processes of learning and memory in the insect nervous system.

Key words: cloned dopamine receptor; Drosophila melanogaster*;* Xenopus *oocyte expression; calcium; adenylyl cyclase; G-protein coupling*

Dopamine is a biogenic amine with a widespread distribution in the insect nervous system, where it has been proposed to function as a neurotransmitter, a neurohormone, and a neuroglandular effector (see Evans, 1980; Brown and Nestler, 1985). Evidence also is accumulating for an important role for dopamine in learning and memory and in neuronal development in insects (Tempel et al., 1984; Budnik and White, 1988; Budnik et al., 1989; Buchner, 1991). However, very little information is available on dopamine receptors and their modes of action in insects.

In the vertebrate nervous system the actions of dopamine are mediated by several pharmacologically distinct subclasses of G-protein-coupled receptors (Jackson and Westlind-Danielsson, 1994; O'Dowd et al., 1994). The general D1-like receptor subclass consists of the cloned receptor subtypes D1 and D5, whereas the D2-like receptor subclass consists of the cloned receptor subtypes, D2, D3, and D4 (Gingrich and Caron, 1993). The D1-like receptors mediate their actions via an activation of adenylyl cyclase activity and phosphatidylinositol 4,5-bisphosphate (PI) metabolism, whereas the D2-like receptors inhibit adenylyl cyclase and activate potassium channels (Gingrich and Caron, 1993; Jackson and Westlind-Danielsson, 1994).

In insects dopamine acts physiologically by activation of D1-like receptors in brain and salivary glands (Evans and Green, 1990a,b; Ali and Orchard, 1994), and D1-like receptor sites are found in the brains of both cockroach (Notman and Downer, 1987) and honey bee (Kokay and Mercer, 1996). Other effects of dopamine in insect brains are mediated by receptors with pharmacologies distinct from those of vertebrate dopamine receptors (Orr et al., 1987; Davis and Pitman, 1991; Kokay and Mercer, 1996). The existence of two dopamine D1-like receptor subtypes in *Drosophila* has been demonstrated by gene cloning (Gotzes et al., 1994; Sugamori et al., 1995; Feng et al., 1996). These receptors have only weak sequence similarity with cloned vertebrate D1-like receptor subtypes. The DopR99B, *Drosophila* D1-like receptor (Feng et al., 1996), may have arisen by a gene duplication from an octopamine/tyramine receptor, OctyR99AB (Arakawa et al., 1990; Saudou et al., 1990). Both of the cloned *Drosophila* D1-like receptors increase adenylyl cyclase activity when expressed in vertebrate cell lines (Gotzes et al., 1994; Sugamori et al., 1995) and *Xenopus* oocytes (Feng et al., 1996). In addition, the activated DopR99B receptor also generates an intracellular Ca^{2+} signal when expressed in *Xenopus* oocytes (Feng et al., 1996). This receptor seems to be expressed preferentially in mushroom bodies in *Drosophila* (Han et al., 1996) and could be involved in the dopaminergic modulation of learning and memory.

To define the way in which the DopR99B *Drosophila* D1-like dopamine receptor may modulate learning and memory, we re-

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port here the mechanisms of its coupling to two second messenger systems when expressed in *Xenopus* oocytes.

Some of these results have been published in abstract form (Evans et al., 1996).

MATERIALS AND METHODS

Synthesis of cRNA. Sense cRNA was prepared *in vitro* from the DopR99B clone (Feng et al., 1996) in pBluescript II SK$^-$ vector with T7 RNA polymerase (Stratagene, Cambridge, UK) after the plasmid was linearized with *Not*I (Promega, Madison, WI). Transcripts were capped by adding 0.75 U of m^{7}G(5′)ppp(5′)G (Boehringer Mannheim UK, Lewes, UK) to a standard 150 μl transcription reaction (Stratagene RNA transcription kit).

The pβART plasmid contains the coding region of the hamster β_2-adrenergic receptor flanked by the 5′ and 3′ untranslated regions of the *Xenopus* β-globin gene and is designed for the production of *in vitro* transcripts that are translated with a high efficiency in *Xenopus* oocytes (White and Reisine, 1990). The pβART plasmid was linearized with *Bam*HI and cRNA transcripts made *in vitro* with SP6 RNA polymerase (White and Reisine, 1990).

Expression in Xenopus *oocytes.* Stage V and VI oocytes from virgin female adult *Xenopus laevis* were separated manually and placed in sterile ND96 medium [(in mM): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, and HEPES buffer 5, pH 7.6, containing 2.4 mM sodium pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.2 mg/ml gentamycin]. The oocytes were defolliculated enzymatically by incubation in ND96 containing collagenase (2 mg/ml) for 30 min. Then the oocytes were injected with 50 ng of either DopR99B receptor cRNA or βART cRNA or both and incubated at 19°C. All oocytes were tested for expression 5 d after the injection of cRNA. Uninjected oocytes were used as controls.

Electrophysiological recordings were made from oocytes by a two-microelectrode voltage-clamp technique, at a −60 mV holding potential, to measure oocyte currents (Van Renterghem et al., 1987). Oocytes were superfused continuously with ND96 during the experiments at room temperature, and drugs were added to the superfusate.

The glutathione *S*-transferase–β-adrenergic receptor kinase 1 C terminus (βARK1-CT GST) fusion protein and the glutathione *S*-transferase (GST) control protein were injected into oocytes to produce the indicated intracellular concentrations 5 min before the start of the experiments. The βARK1-CT GST fusion protein contains residues 646–670 of the βARK1-CT, which are involved in $G_{\beta\gamma}$-subunit binding (Inglese et al., 1993; Koch et al., 1994). This fusion protein blocks the $G_{\beta\gamma}$-mediated effects of muscarinic receptor activation of phospholipase Cβ in *Xenopus* oocytes (Stehno-Bittel et al., 1995).

cAMP assays. To monitor cAMP levels, we preincubated individual oocytes for 30 min in ND96 plus 100 μM isobutylmethylxanthine (IBMX). Experimental oocytes were all incubated for a further 30 min (except for those used in the time course study shown in Fig. 1, for which shorter times also were used) with the desired concentration of agonist in the same medium, while control oocytes (to measure basal cAMP levels) were incubated in parallel in the same medium without agonist. After the incubations each oocyte was homogenized in 500 μl of acidified ethanol and centrifuged to remove particulate matter; the supernatant was evaporated to dryness in a vacuum centrifuge (Savant, Farmingdale, NY). Each sample was taken up in 60 μl of assay buffer and assayed for cAMP with a commercial assay kit (Amersham International).

The mean oocyte basal level of cAMP varied between 0.7 and 2.25 pmol/oocyte for the batches of oocytes from different animals used in the experiments reported in this paper. However, within the batches of oocytes from the same animal the basal levels varied by <15%. The individual experiments reported in this paper were all performed with oocytes from the same batch, and appropriate controls were run for each experiment. The results shown are for typical experiments that were repeated at least three times on different batches of oocytes with the same results. Statistical differences were defined at the level of $p < 0.05$ with hierarchal ANOVA.

Drugs. The drugs used in these experiments were obtained from the following sources: dopamine hydrochloride, (−)-norepinephrine hydrochloride, (−)-epinephrine, tyramine hydrochloride, (±)-*p*-octopamine hydrochloride, IBMX, (±)-isoproterenol hydrochloride, pertussis toxin, phentolamine hydrochloride, and DL-propranolol were from Sigma-Aldrich (Poole, Dorset, UK); *R*(+)-SKF-38393 [*R*(+)-1-phenyl-2,3,4,5-tetrahydro-(^{1}H)-3-benzazepine-7,8-diol], quinelorane dihydrochloride, (−)-quinpirole hydrochloride, PD-128,907 [(+)-(4a*R*,10b*R*)-3,4,4a,10b-tetrahydro-4-propyl-^{2} H,^{5} H-(1) benzopyrano-(4, 3b)-1, 4-oxazin-9-*ol*-hydrochloride], *cis*-(*Z*)-flupentixol dihydrochloride, *R*(+)SCH-23390 [*R* (+) -7-chloro-8-hydroxy-3-methyl-1-phenyl-2, 3, 4, 5-tetrahydro-^{1}H-3-benzazepine hydrochloride], *S*(−)-sulpiride, spiperone hydrochloride, (+)-butaclamol hydrochloride, *S*(−)-eticlopride hydrochloride, domperidone, (+)-bromocriptine methanesulfonate, (±)-6-chloro-APB [(±)-6-chloro-7, 8-dihydroxy-3-allyl-1-phenyl-2, 3, 4, 5-tetrahydro-^{1} H-3-benzazepine hydrobromide], *R*(+)-6-bromo-APB [*R*(+)-6-bromo-7,8-dihydroxy-3-allyl-1-phenyl-2, 3, 4, 5-tetrahydro-^{1} H-3-benzazepine hydrobromide], (±)-6-chloro-PB [(±)-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-^{1}H-3-benzazepine hydrobromide], and (±)-PPHT [(±)-2-(*N*-phenylethyl-*N*-propyl)amino-5-hydroxytetralin hydrochloride] were from Research Biochemicals (Natick, MA); BAPTA-AM was from Calbiochem Novabiochem (Nottingham, UK).

RESULTS

The *Drosophila* dopamine receptor DopR99B, when expressed in *Xenopus* oocytes, couples to both the initiation of an intracellular calcium signal and the stimulation of adenylyl cyclase activity (Feng et al., 1996). To determine whether both of these second messenger events are linked directly to receptor activation, rather than one being a secondary consequence of the other, we have compared the activation characteristics and pharmacology of both of the second messenger responses initiated by this receptor.

Time course of responses

We have monitored the DopR99B-induced changes in intracellular calcium levels by measuring the inward currents generated via activation of the endogenous inward calcium-dependent chloride current in oocytes. This method was pioneered in oocytes expressing G-protein-coupled receptors by Masu et al. (1987). Figure 1*Ai* shows that a 2 min pulse of 1 μM dopamine (a concentration that gives a maximal response; see Feng et al., 1996) initiates a very rapid, transient inward current with an extremely short lag time that peaks within 20 sec of dopamine application. This initial response often decays in the continued presence of dopamine to reveal a second, more variable, slower component of the response that declines to basal levels with a time course of 10–15 min and that frequently has superimposed current oscillations (Fig. 1*Aii*). Similar biphasic time courses for the activation of the calcium-dependent inward chloride current have been described for the activation of a range of G-protein-coupled receptors expressed in *Xenopus* oocytes (Dascal et al., 1986; Yakel et al., 1993). Uninjected control oocytes showed no inward currents in response to dopamine application (data not shown).

The changes in oocyte cAMP levels mediated via the activation of the DopR99B receptor have a much slower time course (Fig. 1*B*) and were obtained by deducting the mean basal levels from the levels in oocytes exposed to dopamine at each time point. Exposure of oocytes expressing this receptor to 10 μM dopamine (a concentration that gives a maximal response; see below) produced time-dependent increases in oocyte cAMP levels after an initial lag period of at least 3 min (Fig. 1*B*). The responses peaked at ~10 min of exposure and remained elevated in the continued presence of dopamine for up to 30 min (the longest exposure time used in the present experiments). Uninjected control oocytes showed no increases in cAMP levels in response to dopamine application (data not shown).

Thus, the DopR99B receptor-initiated changes in intracellular calcium levels have a different time course than the receptor-mediated changes in oocyte cAMP levels. This suggests that an underlying continuous elevation in calcium levels is not required to maintain the changes in oocyte cAMP levels. Nonetheless, it is possible that the rapid initial transient changes in intracellular

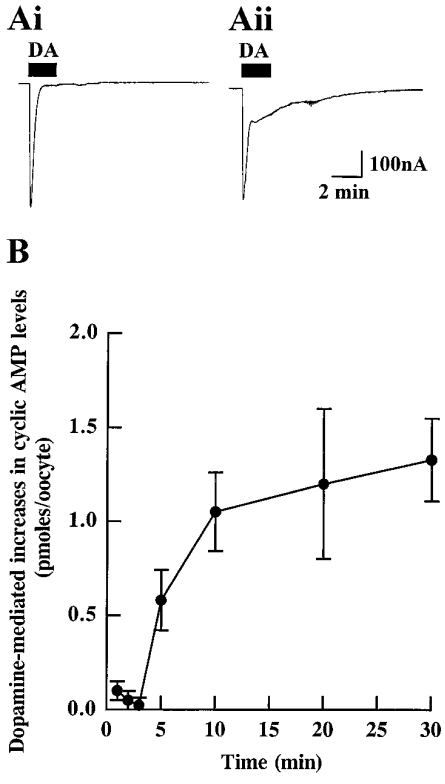

Figure 1. Time courses of second messenger responses. *A,* Typical examples of inward currents generated in response to 2 min pulses of 1 μM dopamine in *Xenopus* oocytes expressing the DopR99B receptor. Responses were obtained 5 d after injection of DopR99B cRNA. *Ai* shows a rapid transient inward current; in addition, *Aii* shows a second, more variable, slower component with superimposed current oscillations. *B,* Time course of the dopamine-mediated increase of cAMP levels in *Xenopus* oocytes expressing the DopR99B receptor. Experimental oocytes were preincubated for 30 min in 100 μM IBMX before exposure to 10 μM dopamine in the presence of 100 μM IBMX for different lengths of time. Control oocytes expressing DopR99B were preincubated as above and then incubated with 100 μM IBMX alone for various lengths of time. The specific dopamine-mediated increase in oocyte cAMP levels was obtained by deducting the mean control values from the experimental values at each time point. The results are expressed as the mean increase in oocyte cAMP levels (pmol/oocyte) ± SE. Oocytes were tested 5 d after injection of cRNA; 10 oocytes were used for each time point.

calcium levels (Fig. 1*Ai*), and/or the more prolonged slower component (Fig. 1*Aii*) are required to initiate changes in oocyte cAMP levels.

Calcium sensitivity of responses

To investigate whether either the changes in intracellular cAMP levels or the inward currents were dependent on extracellular calcium levels, we compared the responses observed in control and in nominally calcium-free media (0 mM Ca^{2+} and 20 mM Mg^{2+}). The elevations in oocyte cAMP levels in response to 10 μM dopamine were not significantly different in control versus nominally calcium-free media (Fig. 2). Similarly, the inward currents generated by oocytes expressing the DopR99B receptor during exposure to pulses of 1 μM dopamine for 2 min were not changed significantly in nominally calcium-free medium (data not shown).

To rule out calcium as an intracellular mediator of the cAMP response, we buffered intracellular calcium levels by a preincubation for 30 min in the presence of the intracellular calcium

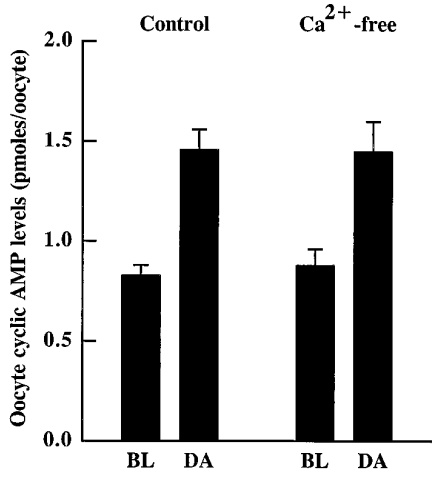

Figure 2. A comparison of the dopamine-induced cAMP response in *Xenopus* oocytes expressing the DopR99B receptor in nominally calcium-free versus control medium. Experimental oocytes (*DA*) were exposed to 10 μM dopamine in the presence of 100 μM IBMX for 30 min after a preincubation for 30 min in 100 μM IBMX alone. The basal oocyte cAMP levels (*BL*) were from oocytes incubated for 60 min in 100 μM IBMX. The results are expressed as the mean oocyte cAMP level (pmol/oocyte) ± SE (10 oocytes).

buffering agent BAPTA-AM (50 μM), and the effects on the cAMP response were measured in oocytes expressing the DopR99B receptor. This treatment did not affect the cAMP response to 10 μM dopamine for 30 min (Fig. 3*A*). In contrast, buffering intracellular calcium substantially reduced or abolished the inward calcium-induced chloride currents in response to 2 min pulses of 1 μM dopamine (Fig. 3*B*).

Thus, the two intracellular messenger effects mediated in *Xenopus* oocytes by activation of the expressed DopR99B receptor can be differentiated by their sensitivity to the release of calcium from intracellular stores, but not on the basis of their sensitivity to changes in extracellular calcium. This suggests that both of the second messenger effects produced by the activation of the receptor by dopamine are direct receptor-mediated events and that the slower changes in oocyte cAMP levels are not produced as a consequence of initial receptor-mediated changes in intracellular calcium levels.

G-protein coupling

Expression of G-protein-coupled receptors in *Xenopus* oocytes indicates that several G-proteins are capable of coupling receptors to the activation of phospholipase C, resulting in the release of calcium from intracellular stores. These include G_s, the classical component of the stimulatory cascade to adenylyl cyclase (De la Penna et al., 1995). We next explored the G-protein coupling of the DopR99B receptor when expressed in *Xenopus* oocytes to see whether different G-protein-mediated pathways are responsible for coupling the receptor to different second messenger pathways.

The dopamine-induced increases in cAMP levels in oocytes expressing the DopR99B receptor are blocked by preexposure of the oocytes to pertussis toxin for either 1 d at 10 μg/ml or 3 d at 0.4 μg/ml (Fig. 4*A*). This suggests the involvement of G_i- or G_o-like G-proteins in this component of the response. In contrast, the inward currents generated by exposure of oocytes to 2 min pulses of 1 μM dopamine are not altered by preexposure to pertussis toxin under either of the two conditions used (Fig. 4*B*).

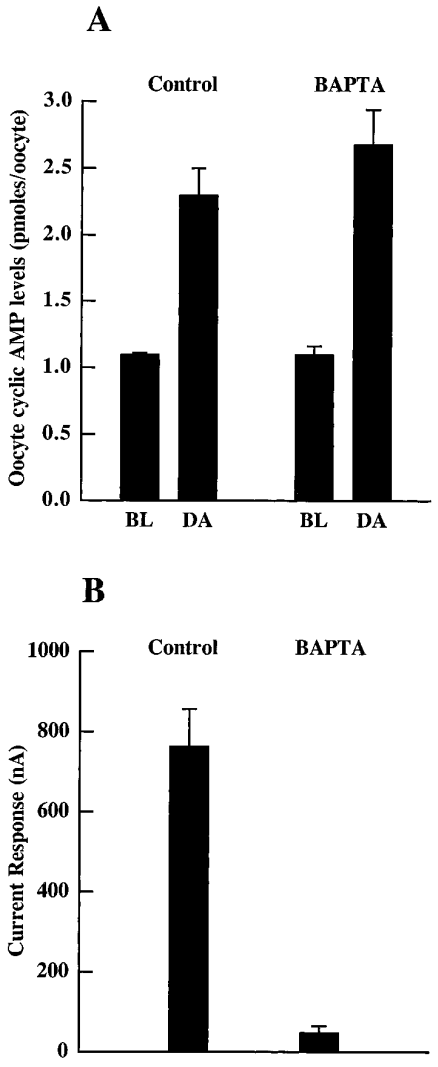

Figure 3. The effect of BAPTA-AM (*BAPTA*) on dopamine-stimulated increases in oocyte cAMP levels (*A*) and on dopamine-mediated inward currents (*B*) in *Xenopus* oocytes expressing the DopR99B receptor. *A*, Experimental oocytes (*DA*) were exposed to 10 μM dopamine in the presence of 100 μM IBMX for 30 min after a preincubation for 30 min in 100 μM IBMX in the presence or absence of 50 μM BAPTA-AM. Basal oocyte cAMP levels (*BL*) were measured after a 30 min exposure to 100 μM IBMX alone after a 30 min preincubation in 100 μM IBMX in the presence or absence of 50 μM BAPTA-AM. The results are expressed as the mean oocyte cAMP levels (pmol/oocyte) ± SE (10 oocytes). *B*, The mean inward current ± SE (10 oocytes) generated by a 2 min exposure to a pulse of 1 μM dopamine after a 30 min preincubation in either control medium or medium containing 50 μM BAPTA-AM.

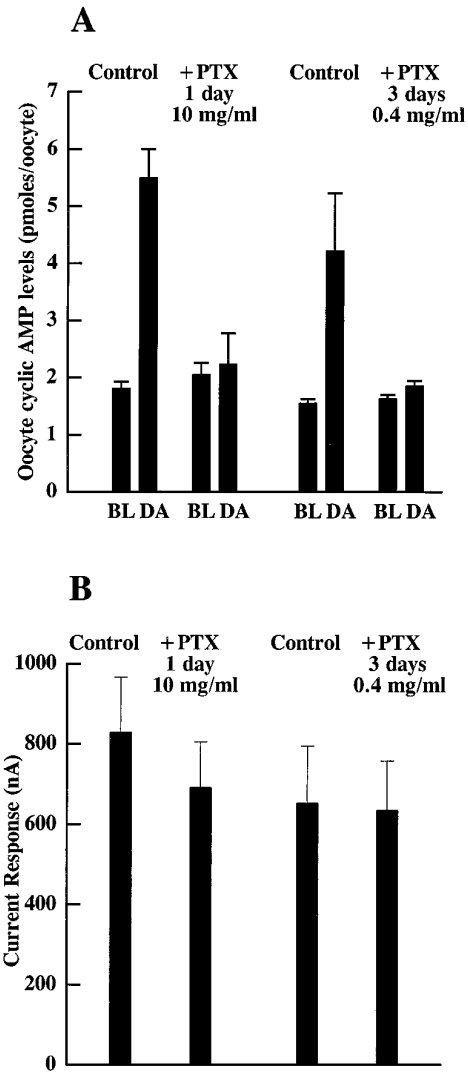

Figure 4. The effect of preexposure to pertussis toxin (*PTX*) on the dopamine-stimulated increases in oocyte cAMP levels (*A*) and on dopamine-mediated inward currents (*B*) in *Xenopus* oocytes expressing the DopR99B receptor. *A*, Oocytes (from different batches) were tested either 1 d after exposure to 10 mg/ml PTX or 3 d after exposure to 0.4 mg/ml PTX. Experimental oocytes (*DA*) were exposed to 10 μM dopamine in the presence of 100 μM IBMX for 30 min after a preincubation for 30 min in 100 μM IBMX alone. Basal oocyte cAMP levels (*BL*) were measured after a 60 min exposure to 100 μM IBMX alone. Control oocytes were not exposed to PTX. The results are expressed as the mean oocyte cAMP levels (pmol/oocyte) ± SE (10 oocytes). *B*, The mean inward current ± SE (10 oocytes) generated by a 2 min exposure to a pulse of 1 μM dopamine after a 1 d exposure to 10 mg/ml PTX, a 3 d exposure to 0.4 mg/ml PTX, or in control oocytes not exposed to PTX. The different PTX exposure regimes were tested on different batches of oocytes.

Thus, the results suggest that the effects of activation of the DopR99B receptor on changes in intracellular calcium and cAMP levels in oocytes are mediated by independent receptor-activated G-protein-coupled pathways.

The involvement of G_i- or G_o-like G-proteins in the dopamine-mediated increases in oocyte cAMP levels in oocytes expressing the DopR99B receptor is unusual, because vertebrate D1-like dopamine receptors are coupled to the activation of adenylyl cyclase activity via a pertussis toxin-insensitive stimulatory G-protein (G_s) (Sibley and Monsma, 1992; Himmler et al., 1993; Ng et al., 1994). However, in many cell types the type II and type IV forms of adenylyl cyclase activity can be stimulated by the $\beta\gamma$ subunits of G-proteins, in addition to $G_{\alpha s}$ (Cooper et al., 1995). Thus, to see whether $\beta\gamma$ subunits are involved in the dopamine-mediated increases in oocyte cAMP levels in oocytes expressing the DopR99B receptor, we have compared the effects of dopamine in the presence and absence of injections of a βARK1-CT GST fusion protein. This fusion protein blocks $G_{\beta\gamma}$ effects because it contains the $G_{\beta\gamma}$-binding region of βARK (Inglese et al., 1993; Koch et al., 1994; Stehno-Bittel et al., 1995). Figure 5 shows that the βARK1-CT GST fusion protein significantly inhibits the dopamine-mediated increase in oocyte cAMP levels at final con-

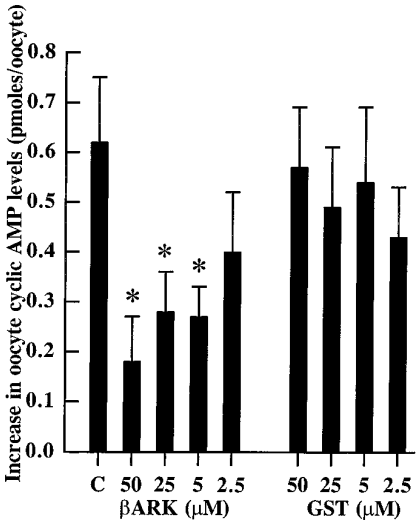

Figure 5. Dose-dependent block of the dopamine-mediated increase in oocyte cAMP levels in oocytes expressing the DopR99B receptor by the βARK1-CT GST fusion protein (*βARK*). Oocytes were exposed to 10 μM dopamine in the presence of 100 μM IBMX for 30 min after a preincubation for 30 min in 100 μM IBMX alone. Control oocytes (*C*) were not injected with any protein. Five minutes before the start of the experiment experimental oocytes were injected with various amounts of either the βARK1-CT GST fusion protein (*βARK*) or a control GST protein (*GST*) to give the indicated intracellular concentrations. The results are expressed as the mean increase in oocyte cAMP levels (pmol/oocyte) ± SE (5 oocytes). *Significantly different from control dopamine responses at the $p < 0.05$ level.

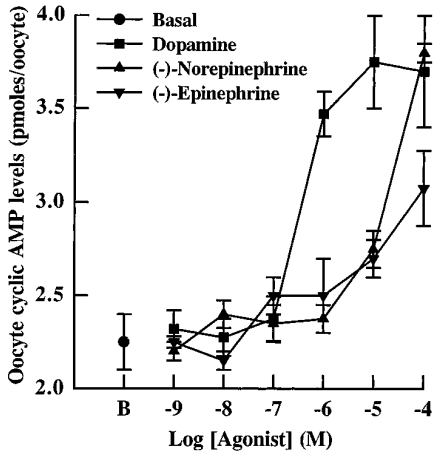

Figure 6. Dose–response curves for the effects of the catecholamines, dopamine, (−)-norepinephrine, and (−)-epinephrine on oocyte cAMP levels in *Xenopus* oocytes expressing the DopR99B receptor. Oocytes were exposed to various concentrations of agonist in the presence of 100 μM IBMX for 30 min after a preincubation for 30 min in 100 μM IBMX alone. Basal oocyte cAMP levels (*B*) were measured after a 60 min exposure to 100 μM IBMX alone. The results are expressed as the mean oocyte cAMP levels (pmol/oocyte) ± SE (10 oocytes).

centrations of 5 μM and above, whereas the control GST segment of the fusion protein alone did not block the effect. In these experiments the dopamine-mediated increases in oocyte cAMP levels were obtained by deducting the mean basal levels from the levels in oocytes exposed to 10 μM dopamine either for groups of uninjected control oocytes or for groups of oocytes injected with different amounts of the βARK1-CT GST fusion protein or the control GST segment of the fusion protein. This result suggests a role for $G_{\beta\gamma}$ subunits in the stimulatory effects of the DopR99B receptor on adenylyl cyclase activity.

$G_{\beta\gamma}$ subunits are also the predominant signaling molecule activating phospholipase C β after the activation of expressed muscarinic M_3 receptors in *Xenopus* oocytes (Stehno-Bittel et al., 1995). Thus, parallel electrophysiology experiments were performed on oocytes expressing the DopR99B receptor in the presence and absence of injections of the βARK1-CT GST fusion protein or the control GST segment of the fusion protein. No significant differences were observed in the inward currents generated by exposure of the oocytes to 2 min pulses of 1 μM dopamine after injection of the βARK1-CT GST fusion protein or the control GST segment of the fusion protein at final concentrations up to 50 μM ($n = 4$; data not shown). Thus, it seems that either $G_{\beta\gamma}$ subunits may not be required for the activation of inward currents by the DopR99B receptor or that the effects of the DopR99B receptor on the stimulation of adenylyl cyclase activity are more sensitive than its effects on the generation of inward currents to the depletion of $G_{\beta\gamma}$ subunits.

Pharmacology of activation of second messenger pathways

Studies on cloned G-protein-coupled receptors show that many receptors potentially can be coupled directly to multiple second messenger systems by independent G-protein-coupled pathways (see Raymond, 1995). It is also clear that ligands differing by as little as a single hydroxyl group can bias the coupling of G-protein-coupled receptors to different second messenger pathways by the process of "agonist-specific coupling" (see Robb et al., 1994; Evans et al., 1995). Because the DopR99B receptor also is coupled to multiple second messenger systems when expressed in *Xenopus* oocytes, we have examined whether it exhibits agonist-specific coupling to these pathways by comparing the pharmacology of its receptor-mediated activation of both intracellular calcium signals and increases in adenylyl cyclase activity.

In a previous study we demonstrated that the catecholamines, dopamine, (−)-norepinephrine, and (−)-epinephrine, but not a range of other biogenic amines, were effective agonists of the receptor-mediated increases in cAMP levels in *Xenopus* oocytes expressing the DopR99B receptor (Feng et al., 1996). Here we present full dose–response curves for the catecholamines. These show that dopamine is two orders of magnitude more potent than either (−)-norepinephrine or (−)-epinephrine (Fig. 6). This is the same order of potency as for the generation of calcium signals by the DopR99B receptor in this preparation (Feng et al., 1996). However, the threshold for an observable increase in oocyte cAMP levels for dopamine occurs between 0.1 and 1 μM, in contrast to our previously observed threshold of between 1 and 10 nM for the receptor-mediated increases in intracellular calcium levels (Feng et al., 1996).

A comparison of the effectiveness of a range of synthetic antagonists to block the dopamine-mediated inward currents and increases in cAMP levels in oocytes expressing the DopR99B receptor is shown in Table 1. In both cases flupentixol was the most effective antagonist tested and *R*(+)-SCH23390, the specific vertebrate D1/D5 dopamine receptor antagonist, was more potent than a range of specific vertebrate D2-like receptor antagonists such as *S*(−)-sulpiride, spiperone, *S*(−)-eticlopride, and domperidone. The α-adrenergic blocker phentolamine and the β-adrenergic blocker DL-propranolol were also poor blockers of both responses, falling within the same range as the weaker

Table 1. Effects of antagonists on inward currents and cAMP responses initiated in *Xenopus* oocytes expressing the *Drosophila* DopR99B receptor

Antagonists (10 μM)	Percentage of inward current* responses to 1 μM dopamine (*n*)	Percentage of cAMP responses to 10 μM dopamine (*n*)	Receptor type specificity
Flupentixol	3.9 ± 1.6 (5)	8.4 ± 3.7 (10)	D1/D2-like
R(+) SCH23390	34.2 ± 8.9 (6)	31.4 ± 9.7 (10)	D1/D5
S(−)-Sulpiride	56.8 ± 16.1 (5)	75.5 ± 6.2 (10)	D2-like
Spiperone	63.0 ± 9.2 (7)	82.3 ± 9.6 (10)	D2-like
(+)-Butaclamol	66.7 ± 4.5 (6)	73.8 ± 7.9 (8)	D2/D1-like
Phentolamine	71.6 ± 7.1 (6)	64.6 ± 14.9 (10)	α-Adrenergic
DL-Propranolol	89.6 ± 5.9 (7)	108.5 ± 22.9 (10)	β-Adrenergic
S-(−)-Eticlopride	103.2 ± 10.9 (4)	103.0 ± 17.6 (10)	D2-like
Domperidone	118.3 ± 7.0 (3)	117.0 ± 18.6 (10)	Peripheral D2

The size of the inward current response to a 2 min pulse of 1 μM dopamine given in the presence of 10 μM antagonist is expressed as a percentage ± SE of the response to a control dopamine pulse given to the same oocyte. The mean response to a 2 min control pulse of 1 μM dopamine was 331.4 ± 15.6 nA ($n = 73$). Antagonists alone did not initiate any currents. The size of the cAMP response to a 30 min exposure to 10 μM dopamine in the presence of 10 μM antagonist and 100 μM IBMX after a 30 min preincubation in the presence of 100 μM IBMX alone is expressed as a percentage ± SE of the response to a control dopamine exposure. The mean response to a 30 min exposure to 10 μM dopamine was an increase of 2.57 ± 0.42 pmol cAMP/oocyte. Antagonists alone did not initiate any cAMP responses.

*Data reproduced from Feng et al. (1996) for comparison.

Table 2. Effects of agonists on inward currents and cAMP responses initiated in *Xenopus* oocytes expressing the *Drosophila* DopR99B receptor

Agonists (10 μM)	Percentage of inward current* response to 1 μM dopamine (*n*)	Percentage of cAMP response to 10 μM dopamine (*n*)	Receptor type specificity
(±)-6-Chloro-APB	89.4 ± 3.8 (6)	1.9 ± 1.2 (10)	D1-like
R(+)-6-Bromo-APB	73.3 ± 9.1 (6)	3.7 ± 2.9 (10)	D1-like
(±)-6-Chloro-PB	19.6 ± 7.7 (5)	13.0 ± 7.6 (10)	D1-like
Quinelorane	11.3 ± 3.0 (8)	3.2 ± 2.1 (10)	D2-like
(±)-Bromocriptine	7.5 ± 2.6 (5)	4.3 ± 1.6 (10)	D2-like
Quinpirole	4.1 ± 2.3 (6)	2.0 ± 0.8 (10)	D2/D3
R(+)-SKF-38393	1.8 ± 1.2 (6)	5.3 ± 1.5 (10)	D1-like
(±) Isoproterenol	1.3 ± 0.9 (6)	3.5 ± 1.5 (10)	β-Adrenergic
PD-128,907	0 (6)	1.3 ± 0.9 (10)	D3
(±)-PPHT	0 (4)	16.1 ± 6.5 (10)	D2-like

The size of the inward current response to a 2 min pulse of agonist is expressed as a percentage ± SE of the response to a control dopamine pulse given to the same oocyte. The size of the cAMP response to a 30 min exposure to 10 μM agonist in the presence of 100 μM IBMX after a 30 min preincubation in the presence of 100 μM IBMX alone is expressed as a percentage ± SE of the response to a control dopamine exposure. All measurements were made 5 d after injection of oocytes with DopR99B cRNA.

*Data reproduced from Feng et al. (1996) for comparison.

D2-like receptor blockers. Thus, in terms of synthetic antagonist responses, both second messenger responses mediated by the DopR99B receptor expressed in *Xenopus* oocytes show an almost identical pharmacological profile similar to vertebrate D1-like dopamine receptors.

In contrast, the pharmacological profile observed for the two receptor-mediated second messenger pathways was different when a range of synthetic agonists was tested. We have shown previously that both D1-like and D2-like agonists could mimic the dopamine-mediated generation of inward currents in oocytes expressing the DopR99B receptor (Feng et al., 1996) and that, with the exception of the relatively ineffective D1-like agonist *R*(+)-SKF-38393, the D1-like agonists were more effective than the D2-like agonists. Table 2, however, shows that the specific D1-like agonists, (±)-6-chloro-APB and *R*(+)-6-bromo-APB, which were the most effective agonists of the generation of inward currents by this receptor, were ineffective agonists of the generation of increases in oocyte cAMP levels. The most effective agonists of the receptor-mediated increases in cAMP levels were (±)-6-chloro-PB, a specific vertebrate D1-like dopamine receptor agonist, and (±)-PPHT, a specific vertebrate D2-like agonist, which was not able to generate inward currents in oocytes expressing the DopR99B receptor. These results suggest that the presence of the allyl grouping at the 3 position of (±)-6-chloro-APB and *R*(+)-6-bromo-APB, which is absent in (±)-6-chloro-PB, is important for the formation of the active configuration of the receptor responsible for coupling the receptor to the pathway responsible for the generation of the inward currents, but it is inhibitory for the coupling of the receptor to the activation of adenylyl cyclase activity. Thus, the DopR99B receptor shows agonist-specific coupling to different second messenger systems for a range of

synthetic agonists, but not for naturally occurring catecholamine ligands.

DISCUSSION

The *Drosophila* D1-like dopamine receptor DopR99B, when expressed in *Xenopus* oocytes, is coupled directly to two independent G-protein-linked pathways. It is coupled to the initiation of an intracellular calcium signal via a pertussis toxin-insensitive pathway and to the stimulation of adenylyl cyclase activity by a pertussis toxin-sensitive pathway. The stimulation of the latter is direct because it does not depend on the generation of an initial calcium signal. Equally, the initial calcium signal does not depend on adenylyl cyclase stimulation because the current response is maximal before any cAMP increases are observed, the current is activated at ~100-fold lower concentrations of dopamine, and the two responses can be activated selectively by different synthetic agonists.

Previous work on vertebrate D1-like dopamine receptors provides convincing evidence for their ability to activate adenylyl cyclase activity directly, but their ability to activate other second messenger pathways or to couple to multiple second messenger pathways directly remains controversial (Jackson and Westlind-Danielsson, 1994; O'Dowd et al., 1994; Kimura et al., 1995). Evidence has been presented that D1-like dopamine receptors can be coupled, in both the brain and the periphery, to other second messenger pathways, including the activation of phospholipase C, the translocation of protein kinase C, the stimulation of K^+ efflux, the inhibition of Na^+/H^+ ATPase activity, and the activation of the arachidonic acid cascade (see Sugamori et al., 1994; Kimura et al., 1995).

Although all of the above effects seem to be independent of adenylyl cyclase activation, it is not clear in many cases if the effects are direct, which subtypes of D1-like receptor mediate the effects, and also if more than one effect can be mediated via stimulation of the same receptor subtype. In particular, the ability of D1-like receptors to stimulate inositol phosphate production and induce intracellular Ca^{2+} mobilization is controversial (see below) and may vary from one cell type to another. Thus, a range of cloned D1-like receptor subtypes did not couple to inositol phosphate effects when expressed in COS-7 (Demchyshyn et al., 1995), baby hamster kidney cells, or Chinese hamster ovary (CHO) (Pedersen et al., 1994) cells, but they did affect calcium metabolism when expressed in Ltk cells (Bouvier et al., 1993). In addition, D1-dopamine receptors coupled to both inositol phosphate production and Ca^{2+} mobilization when rat striatal mRNA was injected into *Xenopus* oocytes, giving inward currents caused by the activation of the endogenous calcium-dependent chloride current, similar to those observed in the present study (Mahan et al., 1990). Further, a cloned, truncated D1-like dopamine receptor from goldfish retina both stimulated cAMP production and increased intracellular calcium mobilization when expressed in HEK 293 cells (Frail et al., 1993). However, in the latter study it was not clear whether both second messenger effects were direct results of receptor activation or whether one of them was a secondary effect. Thus, our data on the expression of the D1-like *Drosophila* dopamine receptor DopR99B in *Xenopus* oocytes are the first evidence for the direct independent coupling of a cloned D1-like dopamine receptor both to the activation of adenylyl cyclase and to the initiation of an intracellular Ca^{2+} signal. The strength of the coupling of the receptor to each of these two second messenger pathways could vary from one neuron to another in the nervous system, depending on their local G-protein environments.

The intracellular Ca^{2+} signals induced by the DopR99B receptor when expressed in *Xenopus* oocytes are likely to be mediated via the activation of G-proteins of the G_q or G_{11} subclasses because they are pertussis toxin-insensitive. Similar pertussis toxin-insensitive activations of phospholipase C leading to intracellular Ca^{2+} signals have been shown previously for the expression of other G-protein-coupled receptors in *Xenopus* oocytes, including the M_3-muscarinic receptor (Stehno-Bittel et al., 1995), the thyrotropin-releasing hormone receptor (Quick et al., 1994; de la Penna et al., 1995), and the neuromedin B receptor (Shapira et al., 1994). This again contrasts with studies on the expression of vertebrate D1-like receptors in rat pituitary GH_4C_1 cells and SK-N-MC neuroblastoma cells in which no evidence of coupling to $G_{q\alpha}$ could be found (Kimura et al., 1995).

Vertebrate D1-like dopamine receptors are coupled to the activation of adenylyl cyclase activity via a pertussis toxin-insensitive stimulatory G-protein (G_s) (Sibley and Monsma, 1992; Himmler et al., 1993; Jackson and Westlind-Danielsson, 1994; Ng et al., 1994; O'Dowd et al., 1994). However, the *Drosophila* DopR99B receptor expressed in *Xenopus* oocytes is coupled to cAMP accumulation via a pathway that is calcium-insensitive but pertussis toxin-sensitive, suggesting the involvement of either G-proteins of the G_i or G_o subclasses. A coupling of DopR99B to G_i seems unlikely because in *Xenopus* oocytes this G-protein is thought to underlie the ability of expressed somatostatin (White and Reisine, 1990) and δ opioid (Tamir and Kushner, 1993) receptors to reduce oocyte cAMP levels in oocytes coexpressing a β_2-adrenergic receptor and stimulated with isoproterenol. In parallel experiments (data not shown) we have not been able to demonstrate a DopR99B receptor-mediated decrease in oocyte cAMP levels after their elevation with isoproterenol after coexpression of a β_2-adrenergic receptor. Thus, we favor the option that a G_o-like G-protein may be involved in mediating this effect of DopR99B receptor activation. Further, it is likely that $G_{\beta\gamma}$-subunits are involved in mediating this response. It is blocked in the presence of the βARK1-CT fusion protein, which selectively binds $G_{\beta\gamma}$-subunits in other preparations (Inglese et al., 1993, 1995; Boekhoff et al., 1994; Koch et al., 1994). Previous studies have shown a $G_{\beta\gamma}$-subunit-mediated stimulation of the type II and type IV forms of adenylyl cyclase activity (Cooper et al., 1995). Another *Drosophila* D1-like receptor has been cloned and expressed in various mammalian cell lines and shown to activate adenylyl cyclase (Gotzes et al., 1994; Sugamori et al., 1995), but nothing is known about its mechanism of adenylyl cyclase activation or of its ability to couple directly or indirectly to multiple second messenger pathways.

The *Drosophila* DopR99B D1-like dopamine receptor exhibits agonist-specific coupling to different second messenger systems (see Evans et al., 1995; Kenakin, 1995, 1996) for a range of synthetic agonists, but not for its known endogenous catecholamine ligands. Thus, the DopR99B receptor exhibits a different synthetic agonist pharmacological profile, depending on which second messenger system is used to assess it. This contrasts with the situation for the cloned *Drosophila* octopamine/tyramine receptor permanently expressed in a CHO cell line (Robb et al., 1994) and for a cloned pituitary adenylyl cyclase-activating peptide (PACAP) type 1 receptor transiently expressed in LLC PK1 kidney cells (Spengler et al., 1993), in which naturally occurring agonists exhibit this effect. The physiological significance of this

phenomenon for the *Drosophila* DopR99B receptor remains unclear at present but raises the possibility that other naturally occurring ligands of this receptor may remain to be found.

The specific coupling of the *Drosophila* DopR99B receptor to different second messenger systems by a range of synthetic agonists, however, parallels that observed for other G-protein-coupled receptors, such as the cloned M_1-muscarinic cholinergic receptor (Gurwitz et al., 1994), the cloned human α_2C10, α_2C4, and α_2C2 adrenergic receptors (Eason et al., 1994), and the cloned 5-HT_{2A} and 5-HT_{2C} receptors (Berg et al., 1995, 1996). In the case of these receptors, there is much interest in the possibility of the generation of new drugs acting via these receptors that might activate only a single desired second messenger pathway and have reduced side effects because of the lack of activation of other undesired pathways. Similarly, agonists could be designed that couple the DopR99B receptor to one or another of the two second messenger pathways that it potentially can activate. Such compounds could lead to the development of highly effective insect control agents, given the preferential expression of this *Drosophila* dopamine receptor in mushroom bodies (Han et al., 1996) and the likely involvement of dopamine in the processes of learning and memory in the insect nervous system (Tempel et al., 1984; Budnik and White, 1988; Schafer and Rehder, 1989; Buchner, 1991; Nassel and Elekes, 1992). Further, genetic studies on dopamine receptor mutants in *Drosophila* could identify the physiological roles of the separate activation of each of the two second messenger systems potentially coupled to the DopR99B receptor, because a variation in the local G-protein environment of different cell types expressing this receptor might allow the receptor to be coupled differently in different cell types.

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